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User Manual

GeneChip® WT PLUS Reagent Kit

Manual Target Preparation for GeneChip® Whole Transcript (WT) Expression Arrays

For Research Use Only. Not for use in diagnostic procedures.

P/N 703174 Rev. 2

Contents

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WT PLUS Reagent Kit

Product Information

Purpose of the Product

The WT PLUS Reagent Kit enables you to prepare RNA samples for whole transcriptome expression analysis with GeneChip® Whole Transcript (WT) Expression Arrays. The kit generates amplified and biotinylated sense-strand DNA targets from total RNA without the need for an up-front selection or enrichment step for mRNA. The kit is optimized for use with GeneChip® Sense Target (ST) Arrays.

The WT PLUS Reagent Kit uses a reverse transcription priming method that primes the entire length of RNA, including both poly(A) and non-poly(A) mRNA to provide complete and unbiased coverage of the transcriptome. The kit is comprised of reagents and a protocol for preparing hybridization-ready targets from 50 to 500 ng of total RNA (Figure 1.1). WT PLUS Reagent is optimized to work with a wide range of samples including tissues, cells, cell lines, and whole blood. The total RNA samples can be used directly without removal of ribosomal or globin RNA prior to target preparation with WT PLUS Reagent.

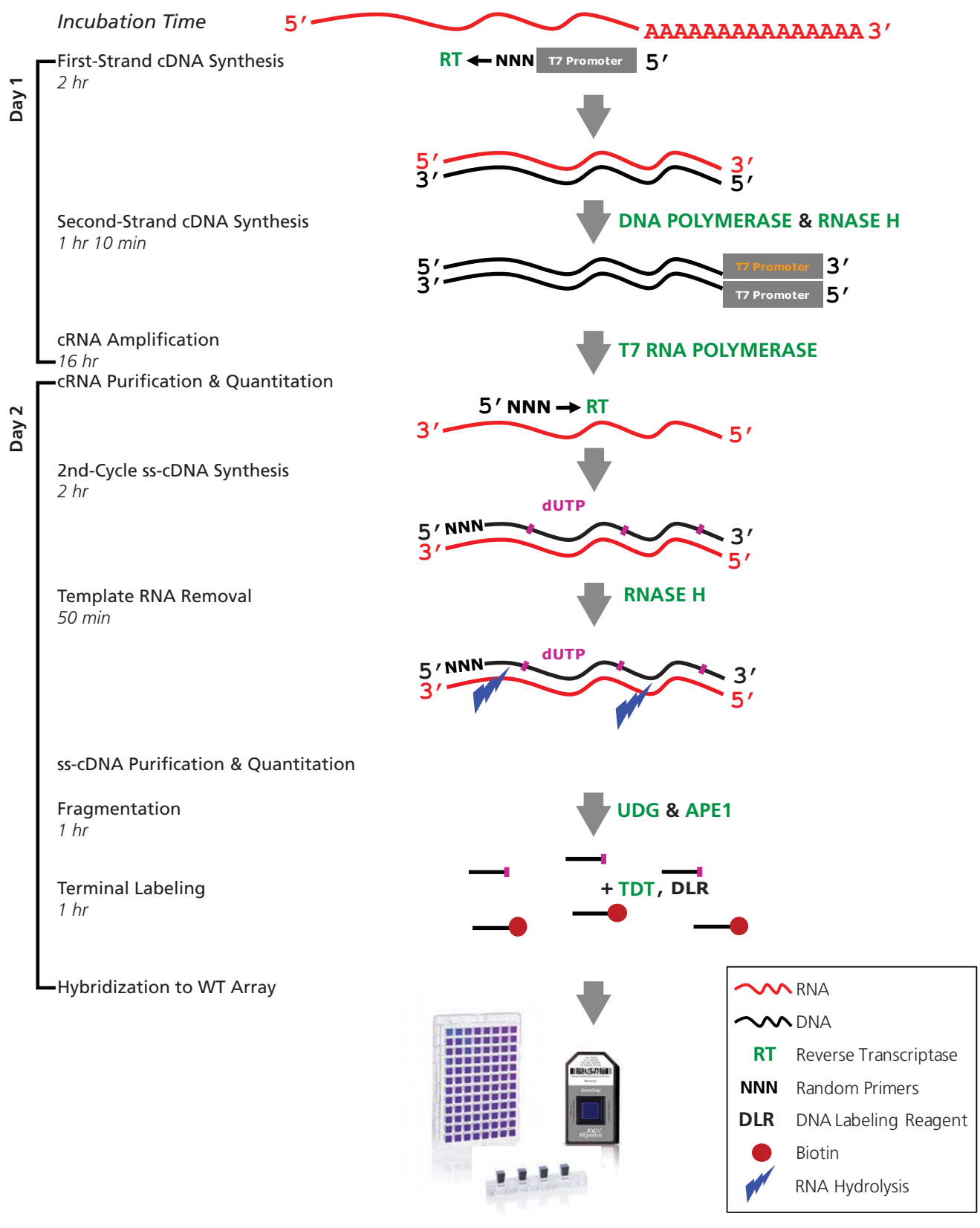
Safety

WARNING: **For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.**

CAUTION: **All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing, such as lab coat, safety glasses and gloves. Care should be taken to avoid contact with skin and eyes. In case of contact with skin or eyes, wash immediately with water. See MSDS (Material Safety Data Sheet) for specific advice.**

Assay Workflow

Figure 1.1 WT PLUS Amplification and Labeling Process



Kit Contents and Storage

Table 1.1 GeneChip® WT PLUS Reagent Kit Contents and Storage

Component	10-Reaction Kit for manual use (P/N 902280)	30-Reaction Kit for manual use (P/N 902281)	Storage
WT Amplification Kit Module 1			
First-Strand Enzyme	11 µL	50 µL	–20°C
First-Strand Buffer	44 µL	160 µL	–20°C
Second-Strand Enzyme	22 µL	70 µL	–20°C
Second-Strand Buffer	198 µL	600 µL	–20°C
IVT Enzyme	66 µL	210 µL	–20°C
IVT Buffer	264 µL	800 µL	–20°C
Control RNA (1 mg/mL HeLa total RNA)	5 µL	5 µL	–20°C
2nd-Cycle Primers	44 µL	180 µL	–20°C
2nd-Cycle ss-cDNA Enzyme	44 µL	140 µL	–20°C
2nd-Cycle ss-cDNA Buffer	88 µL	290 µL	–20°C
RNase H	44 µL	180 µL	–20°C
Nuclease-free Water	2 x 1.0 mL	4 x 1.0 mL	any temp *
WT Amplification Kit Module 2			
Purification Beads	2.2 mL	6.6 mL	4°C †
GeneChip® Poly-A RNA Control Kit			
Poly-A Control Stock	16 µL	16 µL	–20°C
Poly-A Control Dil Buffer	3.8 mL	3.8 mL	–20°C
GeneChip® WT Terminal Labeling Kit			
10X cDNA Fragmentation Buffer	48 µL	213 µL	–20°C
UDG, 10 U/µL	10 µL	49 µL	–20°C
APE 1, 1,000 U/µL	10 µL	49 µL	–20°C
5X TdT Buffer	120 µL	475 µL	–20°C
TdT, 30 U/µL	20 µL	99 µL	–20°C
DNA Labeling Reagent, 5 mM	10 µL	49 µL	–20°C
RNase-free Water	825 µL	2 x 825 µL	any temp *
GeneChip® Expression 3'-Amplification Reagents Hybridization Control Kit			
20X Hybridization Controls	450 µL	450 µL	–20°C
3 nM Control Oligo B2	150 µL	150 µL	–20°C
	**Tubes Organizer:** Plastic vinyl template for organization and storage of components in 9 x 9 array, 81-places square wells, 5 1/4 in. x 5 1/4 in (e.g., Nalgene CryoBox P/N 5026-0909, or equivalent).		

* *Store the Nuclease-free Water at –20°C, 4°C, or room temp.*

† *Do not freeze.*

Required Materials

Instruments

Table 1.2 Instruments Required for Target Preparation

Item	Supplier
Magnetic Stand-96	Agencourt SPRI®Plate Super Magnet Plate (Beckman Coulter Genomics, P/N A32782); Ambion Magnetic Stand-96 (Life Technologies, P/N AM10027); 96-well Magnetic-Ring Stand (Life Technologies, P/N AM10050); or equivalent magnetic stand
Microcentrifuge	Major Laboratory Supplier
NanoDrop® UV-Vis Spectrophotometer	Thermo Scientific, or equivalent quantitation instrument
Optional: 2100 Bioanalyzer	Agilent Technologies, Inc., or equivalent DNA and RNA sizing instrument
Pipette	Major Laboratory Supplier
Thermal Cycler	Various
Vortex Mixer	Major Laboratory Supplier
65°C heat block or oven for incubation of Nuclease-free Water during Purification	Major Laboratory Supplier

Table 1.3 Instruments Required for Array Processing

Instruments	Supplier	Part Number
GeneChip® System for Cartridge Arrays		
GeneChip® Hybridization Oven 645	Affymetrix	P/N 00-0331 (110/220V)
GeneChip® Fluidics Station 450	Affymetrix	P/N 00-0079
GeneChip® Scanner 3000 7G	Affymetrix	P/N 00-0212 (North America) P/N 00-0213 (International)
GeneChip® AutoLoader with External Barcode Reader	Affymetrix	P/N 00-0090 (GCS 3000 7G S/N 501) P/N 00-0129 (GCS 3000 7G S/N 502)
GeneAtlas® System for Array Strips		
GeneAtlas® Workstation	Affymetrix	P/N 90-0894
GeneAtlas® Hybridization Station	Affymetrix	P/N 00-0380 (115VAC) P/N 00-0381 (230VAC)
GeneAtlas® Fluidics Station	Affymetrix	P/N 00-0377
GeneAtlas® Imaging Station	Affymetrix	P/N 00-0376
GeneAtlas® Barcode Scanner	Affymetrix	P/N 74-0015

Table 1.3 Instruments Required for Array Processing (Continued)

Instruments	Supplier	Part Number
GeneTitan® System for Array Plates		
GeneTitan® MC Instrument, NA/Japan includes 110v UPS	Affymetrix	P/N 00-0372
GeneTitan® MC Instrument, Int'l includes 220v UPS	Affymetrix	P/N 00-0373
GeneTitan® Instrument, NA/Japan includes 110v UPS	Affymetrix	P/N 00-0360
GeneTitan® Instrument, Int'l Includes 220v UPS	Affymetrix	P/N 00-0363

Reagents and Supplies

Table 1.4 Additional Reagents and Supplies Required

Item	Supplier
96-well round bottom microtiter plate	Costar, P/N 3795 or equivalent
GeneChip® Hybridization, Wash, and Stain Kit	Affymetrix (P/N 900720, 30 rxns)
GeneAtlas® Hybridization, Wash, and Stain Kit for WT Array Strips	Affymetrix (P/N 901667, 60 rxns)
GeneTitan® Hybridization, Wash and Stain Kit for WT Array Plates	Affymetrix (P/N 901622, 96 rxns)
Nuclease-free aerosol-barrier tips	Major Laboratory Supplier
Nuclease-free 1.5, and 0.2 mL tubes or plates	Major Laboratory Supplier
Nuclease-free 15 mL tubes or containers	Major Laboratory Supplier
Optional: materials for Gel-Shift assay, refer to Appendix A, *Gel-Shift Assay* on page 45	
Optional: RNA 6000 Nano Kit	Agilent Technologies, Inc. P/N 5067-1511; or equivalent DNA and RNA sizing reagents
Tough-Spots®	Major Laboratory Supplier
100% Ethanol (Molecular Biology grade or equivalent)	Major Laboratory Supplier*
Nuclease-free Water (for preparing 80%ethanol wash solution)	Affymetrix (P/N 71786) or major laboratory supplier
Optional: 96-well plate sealing film	Major Laboratory Supplier

* *Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.*

Protocol

Procedural Notes

Implement a Plan to Maintain Procedural Consistency

To minimize sample-to-sample variation that is caused by subtle procedural differences in gene expression assays, consider implementing a detailed procedural plan. The plan standardizes the variables in the procedure and should include the:

- Method of RNA isolation
- Amount of input RNA that is used for each tissue type
- RNA purity and integrity
- *Equipment Preparation*
- Workflow stopping points
- *Reagent Preparation*

Equipment Preparation

Recommended Thermal Cycler

Make sure that the heated cover of your thermal cycler either tracks the temperature of the thermal cycling block or supports specific temperature programming.

Program the Thermal Cycler

Set the temperature for the heated lid to or near the required temperature for each step. An alternate protocol may be used for thermal cyclers that lack a programmable heated lid. This is not the preferred method. Yields of cRNA may be greatly reduced if a heated lid is used during the Second-Strand cDNA Synthesis or during the In Vitro Transcription cRNA Synthesis steps. We recommend leaving the heated lid open during Second-Strand cDNA Synthesis. A small amount of condensation will form during the incubation. This is expected, and should not significantly decrease cRNA yields. For In Vitro Transcription cRNA Synthesis, we recommend incubating the reaction in a 40°C hybridization oven if a programmable heated lid thermal cycler is unavailable.

Incubation temperatures and times are critical for effective RNA amplification. Use properly calibrated thermal cyclers and adhere closely to the incubation times.

NOTE: Concentration fluctuations that are caused by condensation can affect yield. Ensure that the heated lid feature of the thermal cycler is working properly.

Table 2.1 Thermal Cycler Programs

Program	Heated Lid Temp	Alternate Protocol*	Step 1	Step 2	Step 3	Step 4	Volume
First-Strand cDNA Synthesis	42°C	105°C	25°C, 60 min	42°C, 60 min	4°C, 2 min		10 µL
Second-Strand cDNA Synthesis	RT or disable	Lid open	16°C, 60 min	65°C, 10 min	4°C, 2 min		30 µL
In Vitro Transcription cRNA Synthesis	40°C	40°C oven	40°C, 16 hr	4°C, hold			60 µL
2nd-Cycle Primers-cRNA Annealing	70°C	105°C	70°C, 5 min	25°C, 5 min	4°C, 2 min		28 µL
2nd-Cycle ss-cDNA Synthesis	70°C	105°C	25°C, 10 min	42°C, 90 min	70°C, 10 min	4°C, hold	40 µL
RNA Hydrolysis	70°C	105°C	37°C, 45min	95°C, 5 min	4°C, hold		44 µL
Fragmentation	93°C	105°C	37°C, 60 min	93°C, 2 min	4°C, hold		48 µL
Labeling	70°C	105°C	37°C, 60 min	70°C, 10 min	4°C, hold		60 µL
Hybridization Control	65°C	105°C	65°C, 5 min				Variable
Hybridization Cocktail	99°C	105°C	95°C or 99°C, 5 min	45°C, 5 min			Variable

**For thermal cyclers that lack a programmable heated lid.*

Reagent Preparation

Handling kit components as follows:

- Enzymes: Mix by gently vortexing the tube followed by a brief centrifuge to collect contents of the tube, then keep on ice.
- Buffers and Primers: Thaw on ice, thoroughly vortex to dissolve precipitates followed by a brief centrifuge to collect contents of the tube. If necessary, warm the buffer(s) at ≤ 37°C for 1 to 2 min, or until the precipitate is fully dissolved, then keep on ice.
- Purification Beads: Allow to equilibrate at room temperature before use.
- Prepare master mixes for each step of the procedure to save time, improve reproducibility, and minimize pipetting error.
- Prepare Master Mixes as follows:
  - Prepare only the amount needed for all samples in the experiment plus ~5% overage to correct for pipetting losses when preparing the master mixes.
  - Use non-stick nuclease-free tubes to prepare the master mix.
  - Enzyme should be added last and just before adding the master mix to the reaction.
- Return the components to the recommended storage temperature immediately after use.

Prepare Control RNA

Prepare Control RNA

To verify that the reagents are working as expected, a Control RNA sample (1 mg/mL total RNA from HeLa cells) is included with the kit.

To prepare the Control RNA for positive control reaction:

1. On ice, dispense 2 µL of the Control RNA in 78 µL of Nuclease-free Water for a total volume of 80 µL (25 ng/µL).
2. Follow the *Prepare Total RNA/Poly-A RNA Control Mixture* on page 14, but use 2 µL of the diluted Control RNA (50 ng) in the control reaction.

NOTE: The positive control reaction should produce >15 µg of cRNA and >5.5 µg of 2nd-cycle ss-cDNA from 50 ng Control RNA.

Prepare Poly-A RNA Controls

NOTE:

- **To include premixed controls from the GeneChip® Poly-A RNA Control Kit, add the reagents to the total RNA samples. Follow the *Prepare Total RNA/Poly-A RNA Control Mixture* on page 14. Affymetrix strongly recommends the use of Poly-A RNA Controls for all reactions that will be hybridized to GeneChip® arrays.**
- **If frozen, the Poly-A Control Dil Buffer may take 15 to 20 min to thaw at room temperature.**

A set of poly-A RNA controls supplied by Affymetrix is designed specifically to provide exogenous positive controls to monitor the entire target preparation. It should be added to the RNA prior to the First-Strand cDNA Synthesis step.

Each eukaryotic GeneChip® probe array contains probe sets for several *B. subtilis* genes that are absent in eukaryotic samples (*lys*, *phe*, *thr*, and *dap*). These poly-A RNA controls are *in vitro* synthesized, and the polyadenylated transcripts for the *B. subtilis* genes are premixed at staggered concentrations. The concentrated Poly-A Control Stock can be diluted with the Poly-A Control Dil Buffer and spiked directly into RNA samples to achieve the final concentrations (referred to as a ratio of copy number) summarized in Table 2.2.

Table 2.2 Final concentrations of Poly-A RNA Controls when added to total RNA samples

Poly-A RNA Spike	Final Concentration (ratio of copy number)
lys	1:100,000
phe	1:50,000
thr	1:25,000
dap	1:6,667

The controls are then amplified and labeled together with the total RNA samples. Examining the hybridization intensities of these controls on GeneChip® arrays helps to monitor the labeling process independently from the quality of the starting RNA samples.

The Poly-A RNA Control Stock and Poly-A Control Dil Buffer are provided in the GeneChip® Poly-A RNA Control Kit to prepare the appropriate serial dilutions based on Table 2.3. This is a guideline when 50, 100, 250, or 500 ng of total RNA is used as starting material. For starting sample amounts other than those listed here, calculations are needed in order to perform the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

Table 2.3 Serial Dilution of Poly-A RNA Control Stock

Total RNA Input Amount	Serial Dilutions				Volume of 4th Dilution to Add to Total RNA
	1st Dilution	2nd Dilution	3rd Dilution	4th Dilution	
50 ng	1:20	1:50	1:50	1:20	2 µL
100 ng	1:20	1:50	1:50	1:10	2 µL
250 ng	1:20	1:50	1:50	1:4	2 µL
500 ng	1:20	1:50	1:50	1:2	2 µL

IMPORTANT:

- **Avoid pipetting solutions less than 2 µL in volume to maintain precision and consistency when preparing the dilutions.**
- **Use non-stick nuclease-free tubes to prepare all of the dilutions (not included).**
- **After each step, mix the Poly-A Control dilutions thoroughly by gently vortexing followed by a quick centrifuge to collect contents of the tube.**

For example, to prepare the Poly-A RNA dilutions for 100 ng of total RNA:

1. Add 2 µL of the Poly-A Control Stock to 38 µL of Poly-A Control Dil Buffer for the 1st Dilution (1:20).
2. Add 2 µL of the 1st Dilution to 98 µL of Poly-A Control Dil Buffer to prepare the 2nd Dilution (1:50).
3. Add 2 µL of the 2nd Dilution to 98 µL of Poly-A Control Dil Buffer to prepare the 3rd Dilution (1:50).
4. Add 2 µL of the 3rd Dilution to 18 µL of Poly-A Control Dil Buffer to prepare the 4th Dilution (1:10).
5. Add 2 µL of this 4th Dilution to 100 ng of total RNA. The final volume of total RNA with the diluted Poly-A controls should not exceed 5 µL.

TIP: The first dilution of the Poly-A RNA controls can be stored up to 6 weeks in a non-frost-free freezer at –20°C and frozen/thawed up to eight times. Label the storage tube with the expiration date for future reference.

Prepare Total RNA

Evaluate RNA Quality

RNA quality affects how efficiently an RNA sample is amplified using this kit. High-quality RNA is free of contaminating proteins, DNA, phenol, ethanol, and salts. To evaluate RNA quality, determine its A_{260}/A_{280} ratio. RNA of acceptable quality is in the range of 1.7 to 2.1.

Evaluate RNA Integrity

The integrity of the RNA sample, or the proportion that is full length, is an important component of RNA quality. Reverse transcribing partially-degraded mRNA may generate cDNA that lacks parts of the coding region.

Two methods to evaluate RNA integrity are:

- Microfluidic analysis, using the Agilent 2100 Bioanalyzer with an RNA LabChip Kit or equivalent instrument.
- Denaturing agarose gel electrophoresis.

With microfluidic analysis, you use the RNA Integrity Number (RIN) to evaluate RNA integrity. For more information on how to calculate RIN, go to www.genomics.agilent.com

With denaturing agarose gel electrophoresis and nucleic acid staining, you separate and make visible the 28S and 18S rRNA bands. The mRNA is likely to be full length if the:

- 28S and 18S rRNA bands are resolved into two discrete bands that have no significant smearing below each band.
- 28S rRNA band intensity is approximately twice that of the 18S rRNA band.

Determine RNA Quantity

Consider both the type and amount of sample RNA that are available when planning your experiment. Because mRNA content varies significantly with tissue type, determine the total RNA input empirically for each tissue type or experimental condition. The recommended total RNA inputs in Table 2.4 are based on total RNA from HeLa cells. Use these values as reference points for determining your optimal RNA input.

NOTE: Avoid pipetting solutions less than 2 µL in volume to maintain precision and consistency. High-concentration RNA samples should be pre-diluted with Nuclease-free Water before adding to first-strand cDNA synthesis reaction.

Table 2.4 Input RNA Limits

RNA Input	Total RNA
Recommended	100 ng
Minimum	50 ng
Maximum	500 ng

Prepare Total RNA/Poly-A RNA Control Mixture

Prepare total RNA according to your laboratory's procedure. A maximum of 5 µL total RNA can be added to first-strand synthesis reaction. If you are adding Poly-A Spike Controls to your RNA, the volume of RNA must be 3 µL or less (Table 2.5). See *Prepare Poly-A RNA Controls* on page 12 for more information. For example, when performing the Control RNA reaction, combine 2 µL of RNA (25 ng/µL), 2 µL of diluted Poly-A Spike Controls, and 1 µL of Nuclease-free Water.

NOTE: If you are adding Poly-A Spike Controls to your RNA, the volume of RNA must be 3 µL or less. If necessary, use a SpeedVac or ethanol precipitation to concentrate the RNA samples.

Table 2.5 Total RNA/Poly-A RNA Control Mixture

Component	Volume for One Reaction
Total RNA Sample (50-500 ng)	variable
Diluted Poly-A RNA Controls (4th Dilution)	2 µL
Nuclease-free Water	variable
Total Volume	**5 µL**

Synthesize First-Strand cDNA

In this reverse transcription procedure, total RNA is primed with primers containing a T7 promoter sequence. The reaction synthesizes single-stranded cDNA with T7 promoter sequence at the 5' end.

NOTE: Avoid pipetting solutions less than 2 µL in volume to maintain precision and consistency. High-concentration RNA samples should be pre-diluted with Nuclease-free Water before adding to first-strand cDNA synthesis reaction.

1. Prepare First-Strand Master Mix.
   - **A.** On ice, prepare the First-Strand Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare the master mix for all the total RNA samples in the experiment. Include ~5% excess volume to correct for pipetting losses.

Table 2.6 First-Strand Master Mix

Component	Volume for One Reaction
First-Strand Buffer	4 µL
First-Strand Enzyme	1 µL
Total Volume	**5 µL**

   - **B.** Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube. Proceed immediately to the next step.
   - **C.** On ice, transfer 5 µL of the First-Strand Master Mix to each tube or well.
2. Add total RNA to each First-Strand Master Mix aliquot.
   - **A.** On ice, add 5 µL of the total RNA (Table 2.5) to each (5 µL) tube or well containing the First-Strand Master Mix for a final reaction volume of 10 µL.

     See *Prepare Total RNA/Poly-A RNA Control Mixture* on page 14 for more information.
   - **B.** Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.
3. Incubate for 1 hr at 25°C, then for 1 hr at 42°C, then for at least 2 min at 4°C.
   - **A.** Incubate the first-strand synthesis reaction in a thermal cycler using the First-Strand cDNA Synthesis program that is shown in Table 2.1 on page 11.
   - **B.** Immediately after the incubation, centrifuge briefly to collect the first-strand cDNA at the bottom of the tube or well.

C. Place the sample on ice for 2 min to cool the plastic, then proceed immediately to *Synthesize Second-Strand cDNA* on page 16.

IMPORTANT: Transferring Second-Strand Master Mix to hot plastics may significantly reduce cRNA yields. Holding the First-Strand cDNA Synthesis reaction at 4°C for longer than 10 min may significantly reduce cRNA yields.

TIP: When there is approximately 15 min left on the thermal cycler you may start reagent preparation for Second-Strand cDNA Synthesis.

Synthesize Second-Strand cDNA

In this procedure, single-stranded cDNA is converted to double-stranded cDNA, which acts as a template for *in vitro* transcription. The reaction uses DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize second-strand cDNA.

IMPORTANT: Pre-cool thermal cycler block to 16°C.

1. Prepare Second-Strand Master Mix.
   A. On ice, prepare the Second-Strand Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare the master mix for all the first-strand cDNA samples in the experiment. Include ~5% excess volume to correct for pipetting losses.

Table 2.7 Second-Strand Master Mix

Component	Volume for One Reaction
Second-Strand Buffer	18 µL
Second-Strand Enzyme	2 µL
Total Volume	**20 µL**

   B. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube and proceed immediately to the next step.
   C. On ice, transfer 20 µL of the Second-Strand Master Mix to each (10 µL) first-strand cDNA sample for a final reaction volume of 30 µL.
   D. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.
2. Incubate for 1 hr at 16°C, then for 10 min at 65°C, then for at least 2 min at 4°C.
   A. Incubate the second-strand synthesis reaction in a thermal cycler using the Second-Strand cDNA Synthesis program that is shown in Table 2.1 on page 11.

IMPORTANT: Disable the heated lid of the thermal cycler or keep the lid off during the Second-Strand cDNA Synthesis.

   B. Immediately after the incubation, centrifuge briefly to collect the second-strand cDNA at the bottom of the tube or well.
   C. Place the sample on ice, then proceed immediately to *Synthesize cRNA by In Vitro Transcription* on page 17.

TIP: When there is approximately 15 min left on the thermal cycler you may start reagent preparation for In Vitro Transcription.

Synthesize cRNA by In Vitro Transcription

In this procedure, antisense RNA (complimentary RNA or cRNA) is synthesized and amplified by *in vitro* transcription (IVT) of the second-stranded cDNA template using T7 RNA polymerase. This method of RNA sample preparation is based on the original T7 *in vitro* transcription technology known as the Eberwine or RT-IVT method (Van Gelder *et al*., 1990).

IMPORTANT:

- **Transfer the second-strand cDNA samples to room temperature for ≥ 5 min while preparing IVT Master Mix.**
- **After the IVT Buffer is thawed completely, leave the IVT Buffer at room temperature for ≥ 10 min before preparing the IVT Master Mix.**

1. Prepare IVT Master Mix.

   NOTE: This step is performed at room temperature.

   A. At room temperature, prepare the IVT Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare the master mix for all the second-strand cDNA samples in the experiment. Include ~5% excess volume to correct for pipetting losses.

Table 2.8 IVT Master Mix

Component	Volume for One Reaction
IVT Buffer	24 µL
IVT Enzyme	6 µL
Total Volume	**30 µL**

   B. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube, then proceed immediately to the next step.

   C. At room temperature, transfer 30 µL of the IVT Master Mix to each (30 µL) second-strand cDNA sample for a final reaction volume of 60 µL.

   D. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

2. Incubate for 16 hr at 40°C, then at 4°C.

   A. Incubate the IVT reaction in a thermal cycler using the In Vitro Transcription cRNA Synthesis program that is shown in Table 2.1 on page 11.

   B. After the incubation, centrifuge briefly to collect the cRNA at the bottom of the tube or well.

   C. Place the reaction on ice, then proceed to *Purify cRNA* on page 18, or immediately freeze the samples at –20°C for storage.

   TIP: STOPPING POINT. The cRNA samples can be stored overnight at –20°C.

Purify cRNA

In this procedure, enzymes, salts, inorganic phosphates, and unincorporated nucleotides are removed to prepare the cRNA for 2nd-cycle single-stranded cDNA synthesis.

Beginning the cRNA Purification

IMPORTANT:

- **Preheat the Nuclease-free Water in a heat block or thermal cycler to 65°C for at least 10 min.**
- **Mix the Purification Beads thoroughly by vortexing before use to ensure that they are fully dispersed. Transfer the appropriate amount of Purification Beads to a nuclease-free tube or container, and allow the Purification Beads to equilibrate at room temperature. For each reaction, 100 µL plus ~10% overage will be needed.**
- **Prepare fresh dilutions of 80% ethanol wash solution each time from 100% ethanol (Molecular Biology Grade or equivalent) and Nuclease-free Water in a nuclease-free tube or container. For each reaction, 600 µL plus ~10% overage will be needed.**
- **Transfer the cRNA sample to room temperature while preparing the Purification Beads.**

NOTE:

- **Occasionally, the bead/sample mixture may be brownish in color and not completely clear when placed on magnet. In those situations, switch to a different position of magnet on the magnetic stand, a new magnetic stand, or spin out pellets.**
- **This entire procedure is performed at room temperature.**

1. Bind cRNA to Purification Beads.
   - **A.** Mix the Purification Beads container by vortexing to resuspend the magnetic particles that may have settled.
   - **B.** Add 100 µL of the Purification Beads to each (60 µL) cRNA sample, mix by pipetting up and down, and transfer to a well of a U-bottom plate.

     TIP:

     - **Any unused wells should be covered with a plate sealer so that the plate can safely be reused.**
     - **Use multichannel pipette when processing multiple samples.**

   - **C.** Mix well by pipetting up and down 10 times.
   - **D.** Incubate for 10 min. The cRNA in the sample binds to the Purification Beads during this incubation.
   - **E.** Move the plate to a magnetic stand to capture the Purification Beads. When capture is complete (after ~5 min), the mixture is transparent, and the Purification Beads form pellets against the magnets in the magnetic stand. The exact capture time depends on the magnetic stand that you use, and the amount of cRNA generated by *in vitro* transcription.
   - **F.** Carefully aspirate and discard the supernatant without disturbing the Purification Beads. Keep the plate on the magnetic stand.
2. Wash the Purification Beads.
   - **A.** While on the magnetic stand, add 200 µL of 80% ethanol wash solution to each well and incubate for 30 sec.
   - **B.** Slowly aspirate and discard the 80% ethanol wash solution without disturbing the Purification Beads.

C. Repeat Step A and Step B twice for a total of 3 washes with 200 µL of 80% ethanol wash solution. Completely remove the final wash solution.

D. Air-dry on the magnetic stand for 5 min until no liquid is visible, yet the pellet appears shiny. Additional time may be required. Do not over-dry the beads as this will reduce the elution efficiency. The bead surface will appear dull, and may have surface cracks when it is over-dry.

3. Elute cRNA.

A. Remove the plate from the magnetic stand. Add to each sample 27 µL of the preheated (65°C) Nuclease-free Water and incubate for 1 min.

B. Mix well by pipetting up and down 10 times.

C. Move the plate to the magnetic stand for ~ 5 min to capture the Purification Beads.

D. Transfer the supernatant, which contains the eluted cRNA, to a nuclease-free tube.

E. Place the purified cRNA samples on ice, then proceed to *Assess cRNA Yield and Size Distribution*, or immediately freeze the samples at –20°C for storage.

NOTE:

- **Minimal bead carryover will not inhibit subsequent enzymatic reactions.**
- **It may be difficult to resuspend magnetic particles and aspirate purified cRNA when the cRNA is very concentrated. To elute the sample with high concentration cRNA, add an additional 10-30 µL of the preheated Nuclease-free Water to the well, incubate for 1 min, and proceed to Step 3B.**

TIP: STOPPING POINT. The purified cRNA samples can be stored overnight at –20°C. For long-term storage, store samples at –80°C and keep the number of freeze-thaw cycles to 3 or less to ensure cRNA integrity.

Assess cRNA Yield and Size Distribution

Expected cRNA Yield

The cRNA yield depends on the amount and quality of non-rRNA in the input total RNA. Because the proportion of non-rRNA in total RNA is affected by factors such as the health of the organism and the organ from which it is isolated, cRNA yield from equal amounts of total RNA may vary considerably.

During development of this kit, using a wide variety of tissue types, 50 ng of input total RNA yielded 15 to 40 µg of cRNA. For most tissue types, the recommended 100 ng of input total RNA should provide >20 µg of cRNA.

Determine cRNA Yield by UV Absorbance

Determine the concentration of a cRNA solution by measuring its absorbance at 260 nm. Use Nuclease-free Water as blank. Affymetrix recommends using NanoDrop Spectrophotometers for convenience. No dilutions or cuvettes are needed; just use 1.5 µL of the cRNA sample directly. Samples with cRNA concentrations greater than 3,000 ng/uL should be diluted with Nuclease-free Water before measurement and reaction setup. Use the diluted cRNA as the input to prepare 15 µg cRNA in 2nd cycle cDNA synthesis reaction.

Alternatively, determine the cRNA concentration by diluting an aliquot of the preparation in Nuclease-free Water and reading the absorbance in a traditional spectrophotometer at 260 nm. Calculate the concentration in µg/mL using the equation shown below (1 A_{260} = 40 µg RNA/mL).

A_{260} × dilution factor × 40 = µg RNA/mL

(Optional) Expected cRNA Size Distribution

The expected cRNA profile is a distribution of sizes from 50 to 4500 nt with most of the cRNA sizes in the 200 to 2000 nt range. The distribution is quite jagged and does not resemble the profile observed when using a traditional dT-based amplification kit such as 3' IVT Express kit. This step is optional.

Determine cRNA size distribution using a Bioanalyzer.

Affymetrix recommends analyzing cRNA size distribution using an Agilent 2100 Bioanalyzer, a RNA 6000 Nano Kit (PN5067-1511), and mRNA Nano Series II assay. If there is sufficient yield, then load approximately 300 ng of cRNA per well on the Bioanalyzer. If there is insufficient yield, then use as little as 200 ng of cRNA per well. To analyze cRNA size using a Bioanalyzer, follow the manufacturer's instructions.

TIP: STOPPING POINT. The purified cRNA samples can be stored overnight at –20°C.

Synthesize 2nd-Cycle Single-Stranded cDNA

In this procedure, sense-strand cDNA is synthesized by the reverse transcription of cRNA using 2nd-Cycle Primers. The sense-strand cDNA contains dUTP at a fixed ratio relative to dTTP. 15 µg of cRNA is required for 2nd-cycle single-stranded cDNA synthesis.

1. Prepare 15 µg of cRNA.

   On ice, prepare 625 ng/µL cRNA. This is equal to 15 µg cRNA in a volume of 24 µL. If necessary, use Nuclease-free Water to bring the cRNA sample to 24 µL.

   NOTE: High-concentration cRNA samples (> 3000 ng/µL) should be diluted with Nuclease-free Water before measurement and reaction setup. Use the diluted cRNA as the input to prepare 15 µg of cRNA.

2. Prepare cRNA and 2nd-Cycle Primers Mix.
   - **A.** On ice, combine:
     - 24 µL of cRNA (15 µg)
     - 4 µL of 2nd-Cycle Primers
   - **B.** Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube, then proceed immediately to the next step.
3. Incubate for 5 min at 70°C, then 5 min at 25°C, then 2 min at 4°C.
   - **A.** Incubate the cRNA/Primers mix in a thermal cycler using the 2nd-Cycle Primers-cRNA Annealing program that is shown in Table 2.1 on page 11.
   - **B.** Immediately after the incubation, centrifuge briefly to collect the cRNA/Primers mix at the bottom of the tube or well.
   - **C.** Place the mix on ice, then proceed immediately to the next step.
4. Prepare 2nd-Cycle ss-cDNA Master Mix.
   - **A.** On ice, prepare the 2nd-Cycle ss-cDNA Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare the master mix for all the cRNA/Primers samples in the experiment. Include ~5% excess volume to correct for pipetting losses.

Table 2.9 2nd-Cycle ss-cDNA Master Mix

Component	Volume for One Reaction
2nd-Cycle ss-cDNA Buffer	8 µL
2nd-Cycle ss-cDNA Enzyme	4 µL
Total Volume	**12 µL**

B. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube and proceed immediately to the next step.

C. On ice, transfer 12 µL of the 2nd-Cycle ss-cDNA Master Mix to each (28 µL) cRNA/2nd-Cycle Primers sample for a final reaction volume of 40 µL.

D. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

5. Incubate for 10 min at 25°C, then 90 min at 42°C, then 10 min at 70°C, then for at least 2 min at 4°C.

A. Incubate the 2nd-cycle synthesis reaction in a thermal cycler using the 2nd-Cycle ss-cDNA Synthesis program that is shown in Table 2.1 on page 11.

B. Immediately after the incubation, centrifuge briefly to collect the 2nd-cycle ss-cDNA at the bottom of the tube or well.

C. Place the sample on ice and proceed immediately to *Hydrolyze RNA Using RNase H* on page 21.

Hydrolyze RNA Using RNase H

In this procedure, RNase H hydrolyzes the cRNA template leaving single-stranded cDNA.

1. Add RNase H to each 2nd-cycle ss-cDNA sample.

A. On ice, add 4 µL of the RNase H to each (40 µL) 2nd-cycle ss-cDNA sample for a final reaction volume of 44 µL.

B. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

2. Incubate for 45 min at 37°C, then for 5 min at 95°C, then for at least 2 min at 4°C.

A. Incubate the RNA hydrolysis reaction in a thermal cycler using the RNA Hydrolysis program that is shown in Table 2.1 on page 11.

B. Immediately after the incubation, centrifuge briefly to collect the hydrolyzed 2nd-cycle ss-cDNA at the bottom of the tube or well.

C. Place the samples on ice and proceed immediately to the next step.

3. Add Nuclease-free Water to each hydrolyzed 2nd-cycle ss-cDNA sample.

A. On ice, add 11 µL of the Nuclease-free Water to each (44 µL) hydrolyzed 2nd-cycle ss-cDNA sample for a final reaction volume of 55 µL.

B. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the reaction at the bottom of the tube or well.

C. Place the sample on ice, then proceed to *Purify 2nd-Cycle Single-Stranded cDNA* on page 22, or immediately freeze the samples at –20°C for storage.

TIP: STOPPING POINT. The hydrolyzed ss-cDNA samples can be stored overnight at –20°C.

Purify 2nd-Cycle Single-Stranded cDNA

After hydrolysis, the 2nd-cycle single-stranded cDNA is purified to remove enzymes, salts, and unincorporated dNTPs. This step prepares the cDNA for fragmentation and labeling.

Beginning the Single-Stranded cDNA Purification

IMPORTANT:

- **Preheat the Nuclease-free Water in a heat block or thermal cycler to 65°C for at least 10 min.**
- **Mix the Purification Beads thoroughly by vortexing before use to ensure that they are fully dispersed. Transfer the appropriate amount of Purification Beads to a nuclease-free tube or container, and allow the Purification Beads to equilibrate at room temperature. For each reaction, 100 µL plus ~10% overage will be needed.**
- **Prepare fresh dilutions of 80% ethanol wash solution each time from 100% ethanol (Molecular Biology Grade or equivalent) and Nuclease-free Water in a nuclease-free tube or container. For each reaction, 600 µL plus ~10% overage will be needed.**
- **Transfer the cDNA sample to room temperature while preparing the Purification Beads.**

NOTE:

- **Occasionally, the bead/sample mixture may be brownish in color and not completely clear when placed on magnet. In those situations, switch to a different position of magnet on the magnetic stand, a new magnetic stand, or spin out pellets.**
- **This entire procedure is performed at room temperature.**

1. Bind ss-cDNA to Purification Beads.
   - **A.** Mix the Purification Beads container by vortexing to resuspend the magnetic particles that may have settled.
   - **B.** Add 100 µL of Purification Beads to each (55 µL) 2nd-cycle ss-cDNA sample, mix by pipetting up and down, and transfer to a well of a U-bottom plate.

     TIP:

     - **Any unused wells should be covered with a plate sealer so that the plate can safely be reused.**
     - **Use multichannel pipette when processing multiple samples.**

   - **C.** Add 150 µL of 100% ethanol to each (155 µL) ss-cDNA/Beads sample. Mix well by pipetting up and down 10 times.
   - **D.** Incubate for 20 min. The ss-cDNA in the sample binds to the Purification Beads during this incubation.
   - **E.** Move the plate to a magnetic stand to capture the Purification Beads. When capture is complete (after ~5 min), the mixture is transparent, and the Purification Beads form pellets against the magnets in the magnetic stand. The exact capture time depends on the magnetic stand that you use, and the amount of ss-cDNA generated by 2nd-Cycle ss-cDNA Synthesis.
   - **F.** Carefully aspirate and discard the supernatant without disturbing the Purification Beads. Keep the plate on the magnetic stand.
2. Wash the Purification Beads.
   - **A.** While on the magnetic stand, add 200 µL of 80% ethanol wash solution to each well and incubate for 30 sec.

B. Slowly aspirate and discard the 80% ethanol wash solution without disturbing the Purification Beads.

C. Repeat Step A and Step B twice for a total of 3 washes with 200 µL of 80% ethanol wash solution. Completely remove the final wash solution.

D. Air-dry on the magnetic stand for 5 min until no liquid is visible, yet the pellet appears shiny. Additional time may be required. Do not over-dry the beads as this will reduce the elution efficiency. The bead surface will appear dull, and may have surface cracks when it is over-dry.

3. Elute ss-cDNA.

A. Remove the plate from the magnetic stand. Add to each sample 30 µL of the preheated (65°C) Nuclease-free Water and incubate for 1 min.

B. Mix well by pipetting up and down 10 times.

C. Move the plate to the magnetic stand for ~ 5 min to capture the Purification Beads.

D. Transfer the supernatant, which contains the eluted ss-cDNA, to a nuclease-free tube.

E. Place the purified ss-cDNA samples on ice, then proceed to *Assess Single-Stranded cDNA Yield and Size Distribution*, or immediately freeze the samples at –20°C for storage.

NOTE: Minimal bead carryover will not inhibit subsequent enzymatic reactions.

TIP: STOPPING POINT. The purified ss-cDNA samples can be stored overnight at –20°C. For long-term storage at –20°C, we recommend not to proceed to the fragmentation and labeling reaction and store the samples as ss-cDNA.

Assess Single-Stranded cDNA Yield and Size Distribution

Expected Single-Stranded cDNA Yield

During development of this kit, using a wide variety of tissue types, 15 µg of input cRNA yielded 5.5 to 15 µg of ss-cDNA. For most tissue types, the recommended 15 µg of input cRNA should yield > 5.5 µg of ss-cDNA.

Determine Single-Stranded DNA Yield by UV Absorbance

Determine the concentration of a ss-cDNA solution by measuring its absorbance at 260 nm. Use Nuclease-free Water as blank. Affymetrix recommends using NanoDrop Spectrophotometers for convenience. No dilutions or cuvettes are needed; just use 1.5 µL of the cDNA sample directly.

Alternatively, determine the ss-cDNA concentration by diluting an aliquot of the preparation in Nuclease-free Water and reading the absorbance in a traditional spectrophotometer at 260 nm. Calculate the concentration in µg/mL using the equation below (1 A_{260} = 33 µg DNA/mL).

A_{260} × dilution factor × 33 = µg DNA/mL

NOTE: The equation above applies only to single-stranded cDNA.

(Optional) Expected Single-Stranded cDNA Size Distribution

The expected cDNA profile does not resemble the cRNA profile. The median cDNA size is approximately 400 nt. This step is optional.

Determine Single-Stranded cDNA Size Distribution Using a Bioanalyzer

Affymetrix recommends analyzing cDNA size distribution using an Agilent 2100 Bioanalyzer, a RNA 6000 Nano Kit (PN5067-1511), and mRNA Nano Series II assay. If there is sufficient yield, load approximately 250 ng of cDNA per well. If there is insufficient yield, then use as little as 200 ng of cDNA per well. To analyze cDNA size using a bioanalyzer, follow the manufacturer's instructions.

TIP: STOPPING POINT. The purified ss-cDNA samples can be stored overnight at –20°C. For long-term storage at –20°C, we recommend not to proceed to the fragmentation and labeling reaction and store the samples as ss-cDNA.

Fragment and Label Single-Stranded cDNA

In this procedure, the purified, sense-strand cDNA is fragmented by uracil-DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) at the unnatural dUTP residues and breaks the DNA strand. The fragmented cDNA is labeled by terminal deoxynucleotidyl transferase (TdT) using the Affymetrix proprietary DNA Labeling Reagent that is covalently linked to biotin. 5.5 µg of single-stranded cDNA is required for fragmentation and labeling.

1. Prepare 5.5 µg of ss-cDNA.

   On ice, prepare 176 ng/µL ss-cDNA. This is equal to 5.5 µg ss-cDNA in a volume of 31.2 µL. If necessary, use Nuclease-free Water to bring the ss-cDNA sample to 31.2 µL.

2. Prepare Fragmentation Master Mix.

   A. On ice, prepare the Fragmentation Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare the master mix for all the ss-cDNA samples in the experiment. Include ~5% excess volume to correct for pipetting losses.

Table 2.10 Fragmentation Master Mix

Component	Volume for One Reaction
Nuclease-free Water	10 µL
10X cDNA Fragmentation Buffer	4.8 µL
UDG, 10 U/µL	1 µL
APE 1, 1,000 U/µL	1 µL
Total Volume	**16.8 µL**

   B. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube, then proceed immediately to the next step.

   C. On ice, transfer 16.8 µL of the Fragmentation Master Mix to each (31.2 µL) purified ss-cDNA sample for a final reaction volume of 48 µL.

   D. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.

3. Incubate for 1 hr at 37°C, then for 2 min at 93°C, then for at least 2 min at 4°C.

   A. Incubate the fragmentation reaction in a thermal cycler using the Fragmentation program that is shown in Table 2.1 on page 11.

   B. Immediately after the incubation, centrifuge briefly to collect the fragmented ss-cDNA at the bottom of the tube or well.

   C. Place the sample on ice, then proceed immediately to the next step.

4. (Optional) The fragmented ss-cDNA sample can be used for size analysis using a Bioanalyzer. Please see the Reagent Kit Guide that comes with the RNA 6000 Nano LabChip Kit for detailed instructions. The range in peak size of the fragmented samples should be approximately 40 to 70 nt.
5. On ice, transfer 45 µL of the fragmented ss-cDNA sample to each tube or well.
6. Prepare Labeling Master Mix.
   A. On ice, prepare the Labeling Master Mix in a nuclease-free tube. Combine the components in the sequence shown in the table below. Prepare the master mix for all the fragmented ss-cDNA samples in the experiment. Include ~5% excess volume to correct for pipetting losses.

Table 2.11 Labeling Master Mix

Component	Volume for One Reaction
5X TdT Buffer	12 µL
DNA Labeling Reagent, 5 mM	1 µL
TdT, 30 U/µL	2 µL
Total Volume	**15 µL**

   B. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the mix at the bottom of the tube, then proceed immediately to the next step.
   C. On ice, transfer 15 µL of the Labeling Master Mix to each (45 µL) fragmented ss-cDNA sample for a final reaction volume of 60 µL.
   D. Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.
7. Incubate for 1 hr at 37°C, then for 10 min at 70°C, then for at least 2 min at 4°C.
   A. Incubate the labeling reaction in a thermal cycler using the Labeling program that is shown in Table 2.1 on page 11.
   B. Immediately after the incubation, centrifuge briefly to collect the fragmented and labeled ss-cDNA at the bottom of the tube or well.
   C. Place the sample on ice, then proceed to Chapter 3, *WT Array Hybridization* on page 26, or immediately freeze the samples at –20°C for storage.
8. (Optional) Remove 2 µL of each fragmented and labeled ss-cDNA sample for Gel-shift analysis as described in Appendix A, *Gel-Shift Assay* on page 45 to assess the fragmentation and labeling efficiency.

TIP: STOPPING POINT. The fragmented and labeled ss-cDNA samples can be stored overnight at –20°C. For long-term storage at –20°C, we recommend to store the samples as unfragmented and unlabeled ss-cDNA.

WT Array Hybridization

Cartridge Array Hybridization on the GeneChip® Instrument

This section provides instruction for setting up hybridizations for cartridge arrays.

Please refer to *Affymetrix® GeneChip® Fluidics Station 450 User's Guide AGCC* (P/N 08-0295), the *GeneChip® Expression Wash, Stain, and Scan User Manual for Cartridge Arrays* (PN 702731), and the *Affymetrix® GeneChip® Command Console® User Manual* (P/N 702569) for further detail.

Prepare Ovens, Arrays, and Sample Registration Files

1. Turn Affymetrix® Hybridization Oven on, set the temperature to 45ºC and set the RPM to 60. Turn the rotation on and allow the oven to preheat.
2. Equilibrate the arrays to room temperature immediately before use. Label the array with the name of the sample that will be hybridized.
3. Register the sample and array information into AGCC.

Target Hybridization Setup for Cartridge Arrays

Reagents and Materials Required

- GeneChip® Hybridization, Wash and Stain Kit. (Not supplied) For ordering information please refer to Table 1.4 on page 9 or the Affymetrix website.
  - Pre-Hybridization Mix
  - 2X Hybridization Mix
  - DMSO
  - Nuclease-free Water
  - Stain Cocktail 1
  - Stain Cocktail 2
  - Array Holding Buffer
  - Wash Buffer A
  - Wash Buffer B
- GeneChip® Hybridization Control Kit
  - 20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre*)
  - Control Oligonucleotide B2 (3 nM)
- Affymetrix® WT Cartridge Array(s). (Not supplied)

Procedure

1. Prepare Hybridization Master Mix.

   A. At room temperature, thaw the components listed in Table 3.1.

   NOTE: DMSO will solidify when stored at 2-8°C. Ensure that the reagent is completely thawed before use. We recommend to store DMSO at room temperature after the first use.

   B. Heat the 20X Hybridization Controls for 5 min at 65ºC in a thermal cycler using the Hybridization Control program that is shown in Table 2.1 on page 11.

   C. At room temperature, prepare the Hybridization Master Mix in a nuclease-free tube. Combine the appropriate amount of components in the sequence shown in the table below. Prepare the master mix for all the fragmented and biotin-labeled ss-cDNA samples in the experiment. Include ~10% overage to correct for pipetting losses.

Table 3.1 Hybridization Master Mix for a Single Reaction

Component	49 or 64-Format*	100 or 81/4-Format*	169-Format*	Final Concentration
Fragmented and Labeled ss-DNA	**5.2 µg**	**3.5 µg**	**2.3 µg**	**23 ng/µL**
Control Oligo B2 (3 nM)	3.7 µL	2.5 µL	1.7 µL	50 pM
20X Hybridization Controls (*bioB, bioC, bioD, cre*)	11 µL	7.5 µL	5 µL	1.5, 5, 25, and 100 pM respectively
2X Hybridization Mix	110 µL	75 µL	50 µL	1X
DMSO	15.4 µL	10.5 µL	7 µL	7%
Nuclease-free Water	19.9 µL	13.5 µL	9.3 µL	
Total Volume	**160 µL**	**109 µL**	**73 µL**	

* *Please refer to specific probe array package insert for information on array format.*

   D. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the mix and proceed immediately to the next step.

2. Prepare Hybridization Cocktail.

   A. At room temperature, add the appropriate amount of Hybridization Master Mix to each fragmented and biotin-labeled ss-cDNA sample to prepare Hybridization Cocktail.

Table 3.2 Hybridization Cocktail for a Single Array

Component	49 or 64-Format	100 or 81/4-Format	169-Format
Hybridization Master Mix	160 µL	109 µL	73 µL
Fragmented and labeled ss-cDNA	~60 µL* (5.2 µg)	41 µL (3.5 µg)	27 µL (2.3 µg)
Total Volume	**220 µL**	**150 µL**	**100 µL**

* *This volume is 58 µL if a portion of the sample was set aside for gel-shift analysis.*

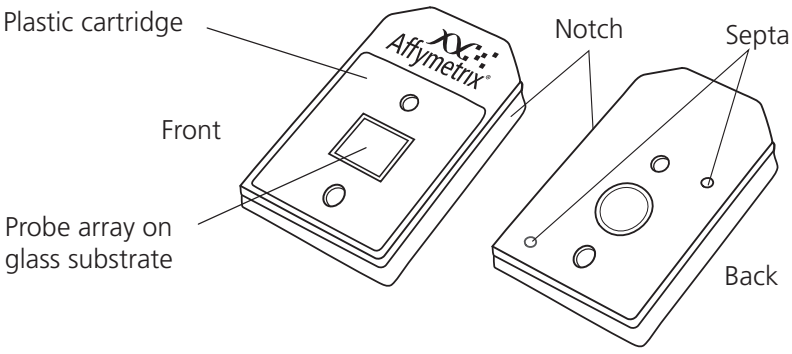
   B. Mix thoroughly by gently vortexing. Centrifuge briefly to collect contents of the tube and proceed immediately to the next step.

   C. Incubate the hybridization cocktail reaction for 5 min at 99°C (tubes) or 95°C (plates), then for 5 min at 45°C in a thermal cycler using the Hybridization Cocktail program that is shown in Table 2.1 on page 11.

D. After the incubation, centrifuge briefly to collect contents of the tube and proceed immediately to the next step.

3. Inject and hybridize array.

Figure 3.1 GeneChip® Probe Array



NOTE: It is necessary to use two pipette tips when filling the probe array cartridge: one for filling and the second to allow venting of air from the hybridization chamber.

A. Insert a pipet tip into the upper right septum to allow for venting.

B. Inject the appropriate amount (see Table 3.3) of the specific sample into the array through one of the septa (see Figure 3.1 for location of the septa on the array).

Table 3.3 Probe Array Cartridge Volumes for Hybridization Cocktail

	49 or 64-Format	100 or 81/4-Format	169-Format
Volume to Load on Array	200 µL	130 µL	80 µL

C. Remove the pipet tip from the upper right septum of the array. Cover both septa with 1/2” Tough-Spots to minimize evaporation and/or prevent leaks.

D. Place the arrays into hybridization oven trays. Load the trays into the hybridization oven.

NOTE: Ensure that the bubble inside the hybridization chamber floats freely upon rotation to allow the hybridization cocktail to make contact with all portions of the array.

E. Incubate with rotation at 60 rpm for 16 hr at 45°C.

NOTE: During the latter part of the 16-hr hybridization prepare reagents for the washing and staining steps required immediately after completion of hybridization.

Wash and Stain

For additional information about washing, staining, and scanning, please refer to the *Affymetrix® GeneChip® Fluidics Station 450 User's Guide AGCC (P/N 08-0295)*, the *GeneChip® Expression Wash, Stain, and Scan User Manual for Cartridge Arrays* (PN 702731), and the *Affymetrix® GeneChip® Command Console® User Manual* (P/N 702569).

1. Remove the arrays from the oven. Remove the Tough-Spots from the arrays.
2. Extract the hybridization cocktail mix from each array. (Optional) Transfer it to a new tube or well of a 96-well plate in order to save the hybridization cocktail mix. Store on ice during the procedure, or at –20ºC for long-term storage.
3. Fill each array completely with Wash Buffer A.
4. Allow the arrays to equilibrate to room temperature before washing and staining.

NOTE: Arrays can be stored in the Wash Buffer A at 4ºC for up to 3 hr before proceeding with washing and staining. Equilibrate arrays to room temperature before washing and staining.

5. Place vials into sample holders on the fluidics station:
   A. Place one (amber) vial containing 600 µL Stain Cocktail 1 in sample holder 1.
   B. Place one (clear) vial containing 600 µL Stain Cocktail 2 in sample holder 2.
   C. Place one (clear) vial containing 800 µL Array Holding Buffer in sample holder 3.
6. Wash the arrays according to array type and components used for Hybridization, Wash and Stain. For HWS kits the protocols are:

Table 3.4 Fluidics Protocol

	49 or 64-Format	100 or 81/4-Format	169-Format
Fluidics Protocol	FS450_0001	FS450_0002	FS450_0007

7. Check for air bubbles. If there are air bubbles, manually fill the array with Array Holding Buffer. If there are no air bubbles, cover both septa with 3/8” Tough-Spots. Inspect the array glass surface for dust and/or other particulates and, if necessary, carefully wipe the surface with a clean lab wipe before scanning.

Scan

The instructions for using the scanner and scanning arrays can be found in the *Affymetrix® GeneChip® Command Console® User Manual* (P/N 702569).

Array Strips Hybridization on the GeneAtlas® Instrument

This section outlines the basic steps involved in hybridizing array strip(s) on the GeneAtlas® System. The two major steps involved in array strip hybridization are:

- *Target Hybridization Setup for Affymetrix® Array Strips* on page 30
- *GeneAtlas® Software Setup* on page 35

NOTE: If you are using a hybridization-ready sample, or re-hybridizing previously made hybridization cocktail, continue the protocol from **Step 5 on page 32**.

IMPORTANT: Before preparing hybridization ready samples, register samples as described in ***GeneAtlas® Software Setup* on page 35**.

Please refer to *GeneAtlas® System User's Guide* (P/N 08-0306) for further detail.

Target Hybridization Setup for Affymetrix® Array Strips

Reagents and Materials Required

- GeneAtlas Hybridization, Wash and Stain Kit for WT Array Strips. (Not supplied) For ordering information please refer to Table 1.4 on page 9 or the Affymetrix website.
  - 5X WT Hyb Add 1
  - 15X WT Hyb Add 4
  - 2.5X WT Hyb Add 6
  - Stain Cocktail 1
  - Stain Cocktail 2
  - Array Holding Buffer
  - Wash Buffer A
  - Wash Buffer B
- GeneChip® Hybridization Control Kit
  - 20X Eukaryotic Hybridization Controls (*bioB*, *bioC*, *bioD*, *cre*)
  - Control Oligonucleotide B2 (3 nM)
- Affymetrix® Array Strip and consumables (Not supplied)
  - Affymetrix WT Array Strip(s)
  - 1 hybridization tray per array strip

Procedure

NOTE: The "WT Hyb Add" reagent names were created to match the order in which reagents are added. For example, WT Hyb Add 1 is the first component added during preparation of the Hybridization Mix. WT Hyb Add 2, 3, and 5 are not used and are not part of the Hybridization Module.

1. In preparation of the hybridization step, prepare the following:
   - **A.** Pull the array strip from storage at 4°C so that it can begin to equilibrate to room temperature.
   - **B.** Gather one (1) hybridization tray per array strip.
   - **C.** Set the temperature of the GeneAtlas Hybridization Station to 48°C. Press the **Start** button.

2. In preparation of the hybridization master mix, prepare the following:
   A. Warm the following vials to room temperature on the bench:
      - 5X WT Hyb Add 1
      - 15X WT Hyb Add 4
      - 2.5X WT Hyb Add 6
   B. Vortex and centrifuge briefly (~5 sec) to collect contents of the tube.
   C. Remove the following tubes from the GeneChip Hybridization Control Kit and thaw at room temperature:
      - Control Oligonucleotide B2 (3 nM)
      - 20X Eukaryotic Hybridization Controls
   D. Vortex and centrifuge briefly (~5 sec) to collect contents of the tube.
   E. Keep the tubes of Control Oligonucleotide B2 (3 nM) and 20X Eukaryotic Hybridization Controls on ice.
3. Prepare the Hybridization Master Mix & Cocktail.
   A. Heat the 20X Hybridization Controls for 5 min at 65ºC in a thermal cycler using the Hybridization Control program that is shown in Table 2.1 on page 11.
   B. At room temperature, prepare the Hybridization Master Mix in a nuclease-free tube. Combine the appropriate amount of components in the sequence shown in the table below. Prepare the master mix for all the fragmented and biotin-labeled ss-cDNA samples in the experiment.

NOTE: The 5X WT Hyb Add 1 solution is very viscous; pipet slowly to ensure addition of the correct volume. Mix well. Vortex and centrifuge briefly (~5 sec) to collect liquid at the bottom of the tube.

Table 3.5 Hybridization Master Mix

Component	Volume for One Array	Volume for Four Arrays (Includes 10% Overage)	Final Concentration
5X WT Hyb Add 1	30 µL	132 µL	1X
Control Oligonucleotide B2 (3 nM)	1.5 µL	6.6 µL	30 pM
20X Hybridization Controls (*bioB, bioC, bioD, cre*)	7.5 µL	33 µL	1.5, 5, 25 and 100 pM, respectively
15X WT Add 4	10 µL	44 µL	1X
Total Volume	**49 µL**	**215.6 µL**	

   C. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the mix and proceed immediately to the next step.
4. Prepare Hybridization Cocktail.
   A. At room temperature, prepare the Hybridization Cocktail in the order as shown in Table 3.6 for all samples.

Table 3.6 Hybridization Cocktail for a Single Array

Component	Volume for One Array	Final Concentration
Hybridization Master Mix	49 µL	
Fragmented and labeled ss-cDNA	41 µL	23 ng/µL
2.5X WT Hyb Add 6	60 µL	1X
Total Volume	**150 µL**	

B. If you are using a plate; seal, vortex, and centrifuge briefly (~5 sec) to collect liquid at the bottom of the well. If you are using tubes; vortex and centrifuge briefly (~5 sec) to collect contents of the tube.

C. Incubate the hybridization cocktail reaction for 5 min at 99°C (tubes) or 95°C (plates), then for 5 min at 45°C in a thermal cycler using the Hybridization Cocktail program that is shown in Table 2.1 on page 11.

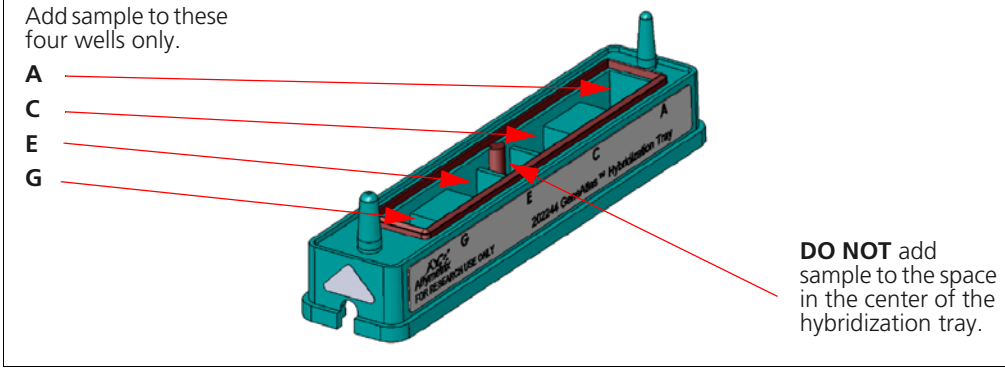
D. After the incubation, centrifuge briefly to collect contents of the tube or well and proceed immediately to the next step.

5. Array Strip Sample Hybridization.

A. Apply 120 µL of hybridization cocktail to the middle of the appropriate wells of a new clean hybridization tray (see Figure 3.2).

IMPORTANT: **Do not add more than 120 µL of hybridization cocktail to the wells as that could result in cross-contamination of the samples.**

Figure 3.2 Location of the sample wells on the hybridization tray



B. Carefully remove the array strip and protective cover from its foil pouch and place on bench (Figure 3.3).

IMPORTANT: **Leave array strip in protective cover.**

Figure 3.3 Array Strip in protective tray

C. Place the array strip into the hybridization tray containing the hybridization cocktail samples (Figure 3.4). Refer to Figure 3.5 for proper orientation of the array strip in the hybridization tray.

Figure 3.4 Placing the array strip into the hybridization tray

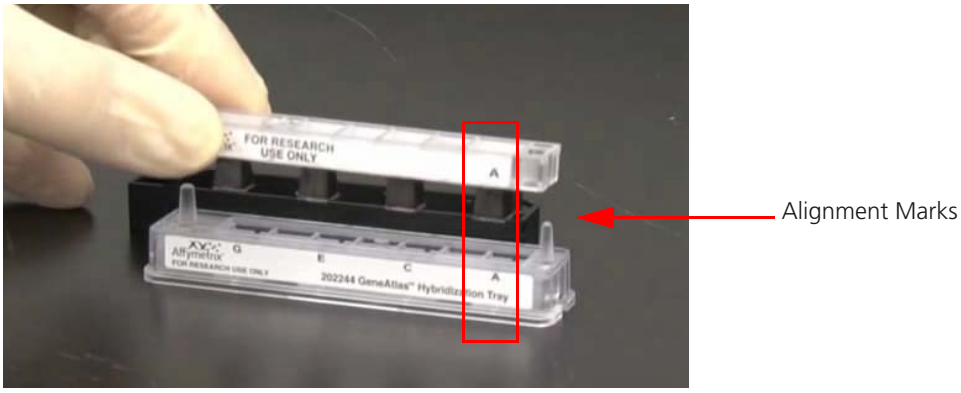
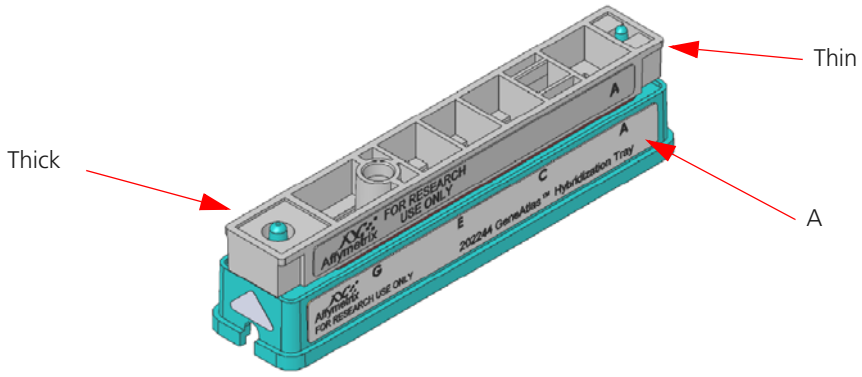


Figure 3.5 Proper Orientation of the Array Strip in the Hybridization Tray



D. Optional: the remainder of the hybridization cocktail Master Mix can be stored at –20°C to supplement Hybridization Cocktail volume should a rehybridization be necessary.

CAUTION: **Be very careful not to scratch/damage the array surface.**

TIP: To avoid any possible mix-ups, the hybridization tray and array strip should be labeled on the white label if more than 1 array strip is processed overnight.

E. Bring the hybridization tray to just above eye level and look at the underside of the hybridization tray to check for bubbles.

CAUTION: Be careful not to tip the hybridization tray to avoid spilling.

IMPORTANT: Insertion of the array strip and air bubble removal should be performed quickly to avoid drying of the array surface.

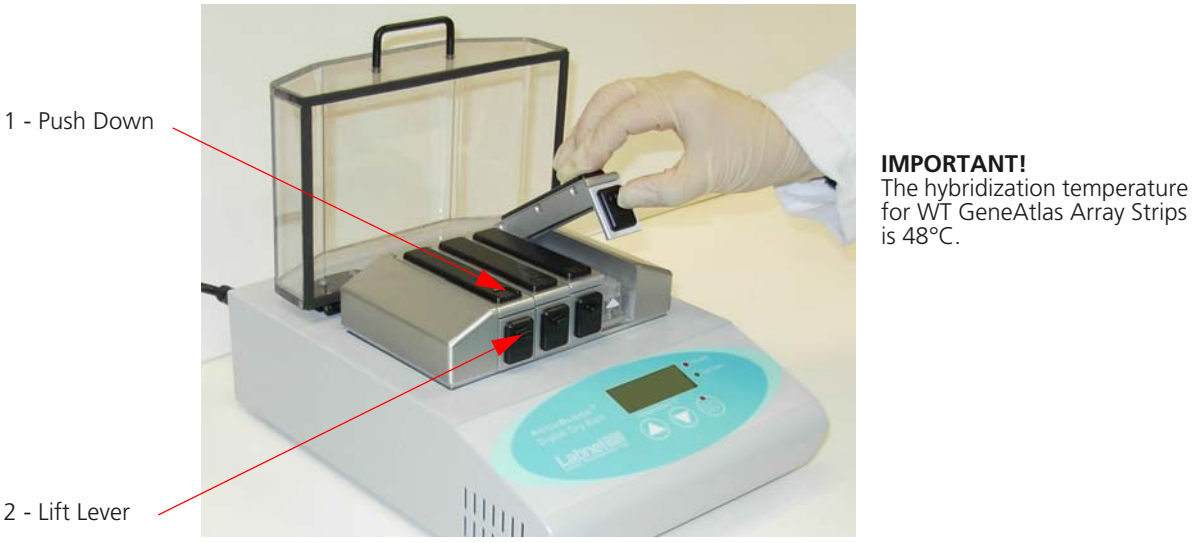
F. If an air bubble is observed, separate the array strip from the hybridization tray and remove air bubbles. Place array strip back into hybridization tray and recheck for air bubbles.

G. Open a Hybridization Station clamp by applying pressure to the top of the clamp while gently squeezing inward. While squeezing lift the clamp to open (Figure 3.6).

WARNING: Do not force the GeneAtlas Hybridization clamps up. To open, press down on the top of the clamp and simultaneously slightly lift the protruding lever to unlock. The clamp should open effortlessly. Refer to **Figure 3.6**.

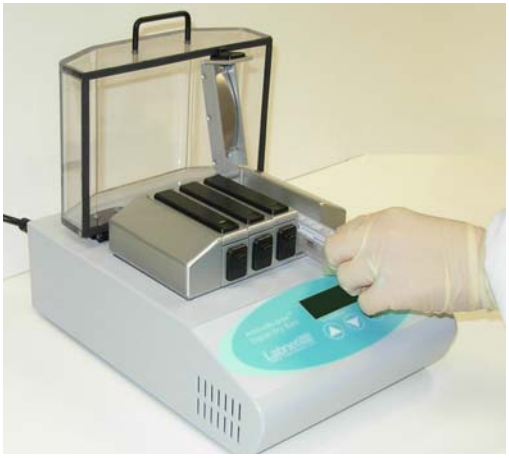
IMPORTANT: The hybridization temperature for WT GeneAtlas Array Strips is 48°C.

Figure 3.6 Opening the clamps on the GeneAtlas® Hybridization Station



H. Place the hybridization tray with the array strip into a clamp inside the Hybridization Station and close the clamp as shown in Figure 3.7.

Figure 3.7 Laterally inserting an array strip and closing the clamp of the GeneAtlas® Hybridization Station



IMPORTANT! The hybridization temperature for WT GeneAtlas Array Strips is 48°C.

6. Proceed to *Hybridization Software Setup* on page 37.

GeneAtlas® Software Setup

Prior to setting up the target hybridization and processing the Affymetrix Array Strips on the GeneAtlas System, each array strip must be registered and hybridizations setup in the GeneAtlas Software.

- **Sample Registration**: Sample registration enters array strip data into the GeneAtlas Software and saves and stores the Sample File on your computer. The array strip barcode is scanned, or entered, and a Sample Name is input for each of the four samples on the array strip. Additional information includes Probe Array Type and Probe Array position.
- **Hybridization Software Setup**: During the Hybridization Software Setup the array strip to be processed is scanned, and the GeneAtlas Hybridization Station is identified with hybridization time and temperature settings determined from installed library files.

For additional information, please refer to *GeneAtlas® System User's Guide* (P/N 08-0306).

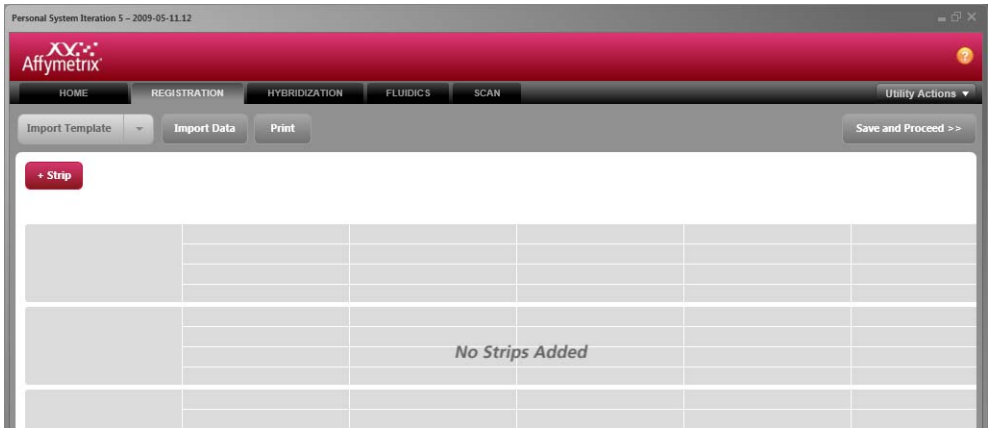
Sample Registration

The following information provides general instructions for registering Affymetrix Array Strips in the GeneAtlas Software. For detailed information on Sample Registration, importing data from Excel and information on the wash, stain and scan steps, please refer to the *GeneAtlas® System User's Guide* (P/N 08-0246).

1. Click **Start** → **Programs** → **Affymetrix** → **GeneAtlas** to launch the GeneAtlas Software.

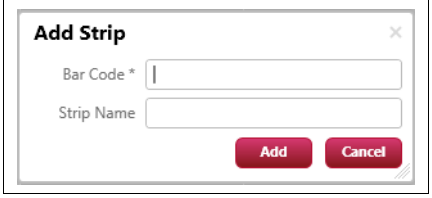
2. Click the **Registration** tab.
   Figure 3.8 appears.

Figure 3.8 Registration Tab of GeneAtlas® Software



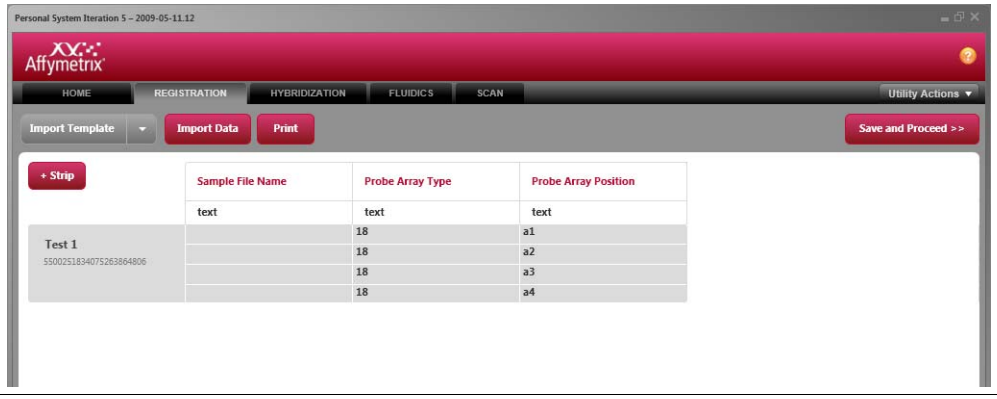
3. Click the **+ Strip** button:  .
   The Add Strip Window appears (Figure 3.9).

Figure 3.9 Add Strip Window



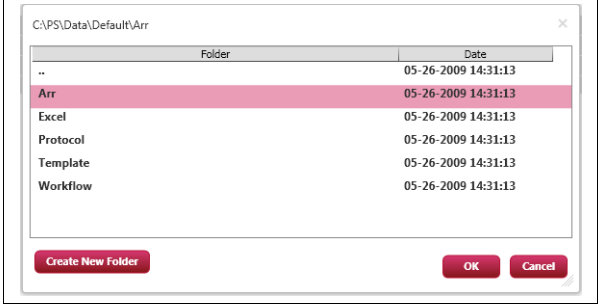
4. Enter or scan the array strip **Bar Code** and enter a **Strip Name**, then click **Add**.
   The array strip is added and appears in the Registration window (Figure 3.10)

Figure 3.10 Array Strip added to Registration window



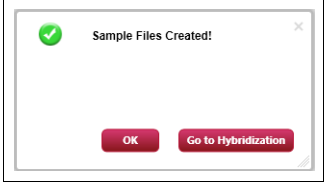
5. Under the **Sample File Name** column, click in the box and enter a sample name and press **Enter**. Enter a unique name for each of the four samples on the array strip.
6. When complete click the **Save and Proceed** button:  .
   The Save dialog box appears (Figure 3.11).

Figure 3.11 Save Dialog



7. In the Save dialog box, click to select a folder in which to save your data. Click **OK**.
   Your files are saved to the selected folder and a confirmation message appears (Figure 3.12).

Figure 3.12



8. Click **OK** to register additional array strips, or click **Go to Hybridization**.

NOTE: You may enter a total of four array strips during the registration process. To add additional strips please repeat Step 3 through Step 8.

9. Proceed to *Hybridization Software Setup* on page 37.

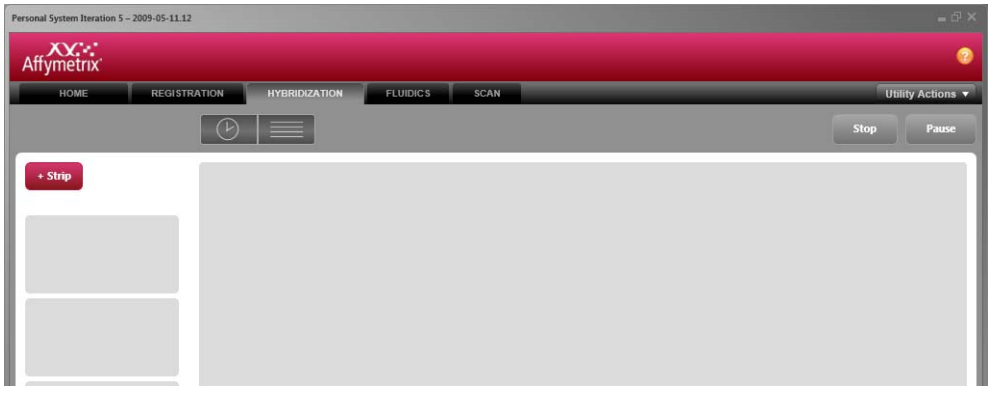
Hybridization Software Setup

All Affymetrix Array Strips to be processed must first be registered prior to setting up the hybridizations in the GeneAtlas Software. Refer to *Sample Registration* on page 35 for instruction on registering array strips.

IMPORTANT: When hybridizing more than one array strip per day, it is recommended to keep the hybridization time consistent. Setup hybridizations for one array strip at a time, staggered by 1.5 hours so that washing and staining can occur immediately after completion of hybridization for each array strip the next day. Recommended hybridization time is 20 ± 1 hour.

1. Navigate to the **Hybridization** tab on the GeneAtlas Software interface.

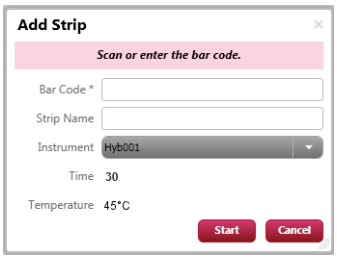
Figure 3.13 Hybridization window



2. Click the **+ Strip** button: 

.
The Add Strip Window appears (Figure 3.14).

Figure 3.14



3. Scan or enter the **Bar Code** (required) of the array strip you registered.
The **Strip Name** field is automatically populated.
4. With the hybridization tray and array strip already in the GeneAtlas Hybridization Station, click **Start** in Figure 3.15.

Figure 3.15

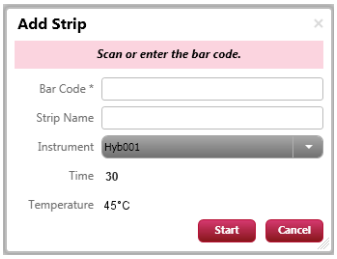
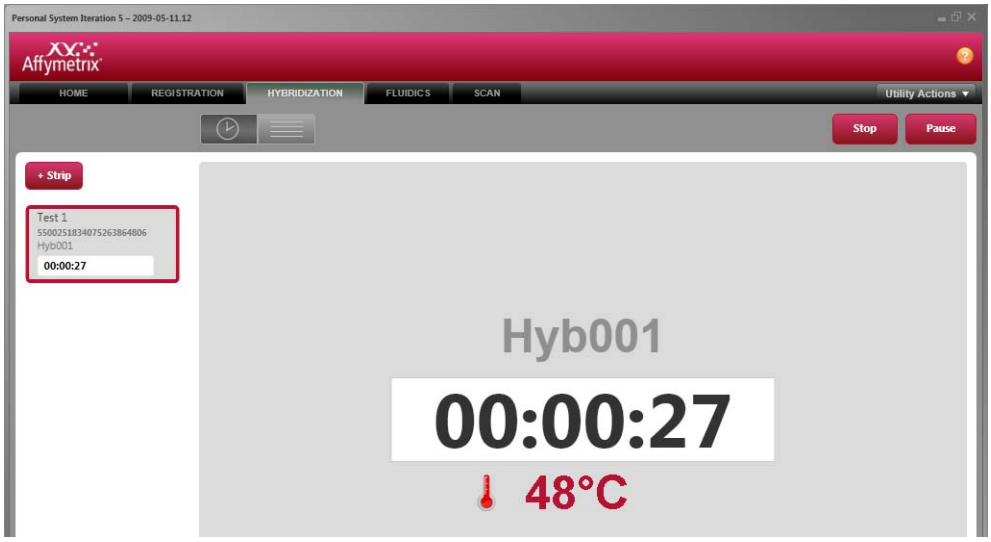
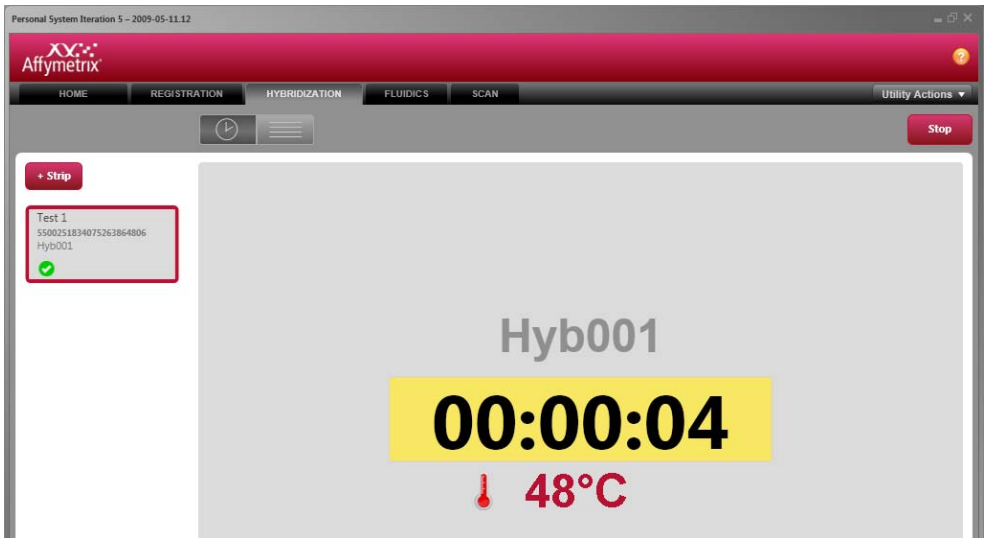


Figure 3.16 Hybridization Countdown



NOTE: The software displays the hybridization time countdown. This time is displayed with a white background (Figure 3.16). When the countdown has completed the display turns yellow and the time begins to count up.

Figure 3.17 Hybridization Count up



5. When hybridization has completed, click the **Stop** button in the upper right corner. A confirmation message box appears (Figure 3.18).

Figure 3.18 Confirmation Message

6. Click **Yes** to complete hybridization.
7. It is important to remove the hybridization tray from the Hybridization Station after the timer has completed the countdown as the Hybridization Station does not shut down when the hybridization is complete.
8. Save the remaining hybridization cocktail in –20°C for future use.
9. Immediately proceed to the GeneAtlas Wash, Stain and Scan protocol. Please refer to *GeneAtlas® System User's Guide* (P/N 08-0306) for further detail.

Array Plates Hybridization on the GeneTitan® Instrument

This chapter outlines the basic steps involved in hybridizing array plate(s) on the GeneTitan® Instrument. The two major steps involved in array plate hybridization are:

- *Target Hybridization Setup for Affymetrix® Array Plates* on page 41
- *Processing WT Array Plates on the GeneTitan® Instrument* on page 43

Please refer to *GeneTitan®Instrument User Guide for Expression Arrays Plates* (P/N 702933) and *Affymetrix® GeneChip® Command Console™ User's Guide* (P/N 702569) for further detail.

Target Hybridization Setup for Affymetrix® Array Plates

Reagents and Materials Required

- GeneTitan® Hybridization, Wash and Stain Kit for WT Array Plates. (Not supplied) For ordering information please refer to Table 1.4 on page 9 or the Affymetrix website.
  - 5X WT Hyb Add 1
  - 15X WT Hyb Add 4
  - 2.5X WT Hyb Add 6
  - Stain Cocktail 1 & 3
  - Stain Cocktail 2
  - Array Holding Buffer
  - Wash Buffer A
  - Wash Buffer B
- GeneChip® Hybridization Control Kit
  - 20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre*)
  - Control Oligonucleotide B2 (3 nM)
- Affymetrix® Array Plate and consumables (Not supplied)
  - Affymetrix® WT Array Plate(s)

Procedure

NOTE: The "WT Hyb Add" reagent names were created to match the order in which reagents are added. For example, WT Hyb Add 4 is the fourth component added during preparation of the Hybridization Mix. WT Hyb Add 2, 3 and 5 are not used and are not part of the Hybridization Module.

1. In preparation of the hybridization step, prepare the following:
   - **A.** Warm the following vials to room temperature on the bench:
     - 5X WT Hyb Add 1
     - 15X WT Hyb Add 4
     - 2.5X WT Hyb Add 6.
   - **B.** Vortex and centrifuge briefly (~5 sec) to collect contents of the tube.
   - **C.** Remove the following tubes from the GeneChip Hybridization Control Kit and thaw at room temperature:
     - Control Oligonucleotide B2 (3 nM)
     - 20X Eukaryotic Hybridization Controls
   - **D.** Vortex and centrifuge briefly (~5 sec) to collect liquid at the bottom of the tube.
   - **E.** Keep the tubes of Control Oligonucleotide B2 (3 nM) and the tube of 20X Eukaryotic Hybridization Controls on ice.

2. Prepare the WT Hybridization Master Mix & Cocktail.

   A. Heat the 20X Hybridization Controls for 5 min at 65ºC in a thermal cycler using the Hybridization Control program that is shown in Table 2.1 on page 11.

   B. At room temperature, prepare the Hybridization Master Mix in a nuclease-free tube. Combine the appropriate amount of components in the sequence shown in the table below. Prepare the master mix for all the fragmented and biotin-labeled ss-cDNA samples in the experiment.

NOTE: The 5X WT Hyb Add 1 solution is very viscous; pipet slowly to ensure addition of the correct volume. Mix well. Vortex and centrifuge briefly (~5 sec) to collect liquid at the contents of the tube.

Table 3.7 Hybridization Master Mix

Component	Volume for One Array	16-Array Plate*	24-Array Plate*	96-Array Plate*	Final Concentration
5X WT Hyb Add 1	24 µL	422.4 µL	633.6 µL	2,534.4 µL	1X
Control Oligo B2 (3 nM)	1.2 µL	21.1 µL	31.7 µL	126.7 µL	30 pM
20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre*)	6 µL	105.6 µL	158.4 µL	633.6 µL	1.5, 5, 25 and 100 pM, respectively
15X WT Hyb Add 4	8 µL	140.8 µL	211.2 µL	844.8 µL	1X
Total Volume	**39.2 µL**	**689.9 µL**	**1,034.9 µL**	**4,139.5 µL**	

* *Includes ~ 10% overage to cover pipetting error.*

   C. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the mix and proceed immediately to the next step.

3. Prepare Hybridization Cocktail.

   A. At room temperature, prepare the Hybridization Cocktail in the order as shown in Table 3.8 for all samples.

Table 3.8 Hybridization Cocktail for a Single Array

Component	Volume for One Array	Final Concentration
Hybridization Master Mix	39.2 µL	
Fragmented and labeled ss-cDNA	32.8 µL	23 ng/µL
2.5X WT Hyb Add 6	48 µL	1X
Total Volume	**120 µL**	

   B. If you are using a plate; seal, vortex, and centrifuge briefly (~5 sec) to collect liquid at the bottom of the well. If you are using 1.5 mL tubes; vortex and centrifuge briefly (~5 sec) to collect contents of the tube.

   C. Incubate the hybridization cocktail reaction for 5 min at 99°C (tubes) or 95°C (plates), then for 5 min at 45°C in a thermal cycler using the Hybridization Cocktail program that is shown in Table 2.1 on page 11.

   D. After the incubation, centrifuge briefly to collect contents of the tube or well and proceed immediately to the next step.

   E. Place 90 µL of the centrifuged supernatant hybridization cocktail as indicated into the appropriate well of the hybridization tray.

   F. Proceed to *Hybridization Setup* on page 43.

Hybridization Setup

This section describes the GeneTitan Setup protocol for WT Array Plates. The reagent consumption per process on the GeneTitan® Instrument for processing WT Array Plates is shown in Table 3.10.

Table 3.9 The Minimum Volumes of Buffer and Rinse Required to Process on the GeneTitan Instrument

Fluid Type	Amount Required for One Array Plate	Minimum Level in Bottle	
		One Array Plate	Two Array Plates
Rinse	300 mL	450 mL	900 mL
Wash A	~920 mL	1,040 mL +	2,000 mL
Wash B	300 mL	450 mL	600 mL

Table 3.10 Volumes Required to Process WT Array Plates per Run

Reagent	Amount Required for One Array Plate	Number of Plates that can be Processed using the GeneTitan Hybridization, Wash and Stain Kit for WT Array Plates (P/N 901622)		
		16-Format	24-Format	96-Format
Wash A	~920 mL	1	1	1
Wash B	300 mL	1	1	1
Stain 1 and 3	105 µL/well	6	4	1
Stain 2	105 µL/well	6	4	1
Array Holding Buffer	150 µL/well	6	4	1

IMPORTANT: The instrument must have a minimum of 450 mL of Wash B in the Wash B reservoir of the instrument for each WT Array Plate prior to starting Hyb, Wash, Stain and Scan process. The waste bottle should be empty.

Processing WT Array Plates on the GeneTitan® Instrument

1. Use the anti-static gun on the wells of the stain tray labeled GeneTitan Stain Tray P/N 501025.
   - **A.** Place a stain tray on the table top.
   - **B.** Hold the Zerostat 3 anti-static gun within 12” (30.5 cm) of the surface or object to be treated. Squeeze the trigger slowly for about two seconds, to emit a stream of positive ionized air over the surface of the object. As the trigger is slowly released, a negative flow of air ions is produced resulting in static neutralization.
   - **C.** Repeat this procedure at several points across the surface of the stain tray.
2. Aliquot 105 µL of the Stain 1 into the GeneTitan Stain Tray.
3. Use the anti-static gun on the stain tray cover.
   - **A.** Place a stain tray cover on the table top with the flat surface facing upward.
   - **B.** Hold the Zerostat 3 anti-static gun within 12" (30.5 cm) of the surface or object to be treated. Squeeze the trigger slowly for about two seconds, to emit a stream of positive ionized air over the surface of the object. As the trigger is slowly released, a negative flow of air ions is produced resulting in static neutralization.
   - **C.** Repeat this procedure at several points across the surface, covering the entire stain tray cover.

4. After removing the static electricity, place the cover on top Stain Tray 1.
5. After repeating Step 1, aliquot 105 µL of the Stain 2 into the GeneTitan Stain Tray.
6. After repeating Step 3, place cover on top of Stain Tray 2.
7. After repeating Step 1, aliquot 105 µL of the Stain 3 into the GeneTitan Stain Tray.
8. After repeating Step 3, place cover on top of Stain Tray 3.
9. Aliquot 150 µL of the Array Holding Buffer into the GeneTitan Scan Tray identified with the label HT Scan Tray P/N 500860 on the tray.
10. Use the fourth scan tray cover provided with the GeneTitan Consumable Upgrade kit to cover the Scan Tray.
11. Load all the consumables including the HT Array Plate into the GeneTitan Instrument as per instructions provided in the *GeneTitan®Instrument User Guide for Expression Arrays Plates* (P/N 702933).

IMPORTANT: **It is important not to bump the trays while loading them into the GeneTitan Instrument. Droplets of the stain going onto the lid may result in a wicking action and the instrument gripper may be unable to remove the lids properly.**

The remaining hybridization ready sample can be stored at –20°C after the Biorad Hardshell Plate using Aluminum Foil.

Gel-Shift Assay

The efficiency of the labeling procedure can be assessed using the following procedure. This quality control protocol prevents hybridizing poorly labeled target onto the probe array. The addition of biotin residues is monitored in a gel-shift assay, where the fragments are incubated with avidin prior to electrophoresis. The nucleic acids are then detected by staining. The procedure takes approximately 90 min to complete.

Table A.1 Additional Reagents Required

Item	Supplier	P/N
XCell SureLock® Mini-Cell*	Life Technologies	EI0001
4-20% TBE Gel1.0 mm, 12 well*	Life Technologies	EC62252BOX
Novex® Hi-Density TBE Sample Buffer (5X)	Life Technologies	LC6678
TBE Buffer, 5X Solution	Affymetrix	75891
SYBR® Gold Nucleic Acid Gel Stain	Life Technologies	S11494
10 bp DNA ladder and 100 bp DNA ladder	Life Technologies	10821-015 and 15628-019
NeutrAvidin Protein	Thermo Scientific	31000
PBS, pH 7.2	Life Technologies	20012-027

**Or equivalent.*

NOTE: Place a 4% to 20% TBE gel into the gel holder and add 1X TBE Buffer to the gel system and equilibrate to room temperature.

1. Prepare NeutrAvidin and biotin-labeled cDNA sample mix.
   - **A.** On ice, prepare a NeutrAvidin solution of 2 mg/mL in PBS.
   - **B.** For each sample to be tested, prepare 2 aliquots of 1 µL fragmented and biotin-labeled ss-cDNA sample in a tube or well.
   - **C.** Heat the samples for 2 min at 70ºC. Centrifuge briefly to collect the reaction at the bottom of the tube or well, then proceed immediately to the next step.
   - **D.** At room temperature, add 5 µL of the 2 mg/mL NeutrAvidin solution to one tube or well and add 5 µL of PBS to the other tube or well.
   - **E.** Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect contents of the tube, and incubate for 5 min at room temperature.
2. Separate the fragmented and labeled ss-cDNA by size and stain.
   - **A.** Prepare 10 bp and 100 bp DNA ladders by combining 1 µL of ladder and 7 µL of Nuclease-free Water.
   - **B.** At room temperature, add loading dye to all samples and DNA ladders to a final concentration of 1X loading dye.
   - **C.** Mix thoroughly by gently vortexing the tube. Centrifuge briefly to collect contents of the tube, and proceed immediately to the next step.
   - **D.** Carefully load samples and ladders on gel. Each well can hold a maximum of 20 µL.

E. Run the gel at 150 volts until the front dye almost reaches the bottom, approximately 1 hr.

F. While the gel is running, prepare 100 mL of a 1X solution of SYBR Gold for staining. SYBR Gold may be diluted in 1X TBE running buffer or water.

NOTE: SYBR Gold is light sensitive. Therefore, use caution and shield the staining solution from light. Prepare a new batch of stain at least once a week.

G. After the gel is complete, break open cartridge and stain the gel in 1X SYBR Gold for 10 min at room temperature.

3. Place the gel on a UV light box and image using the appropriate filter for SYBR Gold.

4. The absence of a shift pattern indicates poor biotin labeling. The problem should be addressed before proceeding to the hybridization step.

Troubleshooting

Observation	Possible Cause	Solution
The positive control sample and your total RNA sample yield low levels of amplified cRNA product or low levels of appropriately sized cRNA product.	Incubation temperatures are incorrect or inaccurate.	Calibrate your thermal cycler.
	Condensation formed in the tubes during the incubations.	Check that the heated lid is working correctly and is set to the appropriate temperature.
	cRNA purification is not performed properly.	Perform the purification as described in this manual.
	Pipettes, tubes, and/or equipment are contaminated with nucleases.	Remove RNases and DNases from surfaces using RNase decontamination solution.
The positive control sample produces expected results, but your total RNA sample results in low levels of amplified cRNA/cDNA product.	The input total RNA concentration is lower than expected.	Repeat the A_{260} reading of your RNA sample. Use 100 to 200ng of total RNA in the First-Strand cDNA Synthesis procedure.
	Your input RNA contains contaminating DNA, protein, phenol, ethanol, or salts, causing inefficient reverse transcription.	Phenol extract and ethanol precipitate your total RNA.
The positive control sample produces expected results but your total RNA sample results in low levels of appropriately sized cRNA/cDNA product.	The total RNA integrity is partially degraded, thereby generating short cDNA fragments.	Assess the integrity of your total RNA sample by determining the size of the 18S and 28S rRNA bands and the relative abundance of 28S to 18S rRNA. Refer to *Evaluate RNA Integrity* on page 13.
	The mRNA content of your total RNA sample is lower than expected.	Verify the mRNA content of your total RNA. Note: In healthy cells, mRNA constitutes 1 to 10% of total cellular RNA (Johnson, 1974; Sambrook and Russel, 2001).

References

Johnson, L.F., Abelson, H.T., Green, H., and S. Penman. 1974. Cell 1:95–100.

Sambrook, J. and D.W. Russel. 2001. Extraction, purification, and analysis of mRNA from eukaryotic cells. In: Molecular cloning, a laboratory manual, third edition, Vol 1. Cold Spring Harbor, New York: Cold Spring Harbor Press.

Van Gelder, R.N., von Xastrow, M.E., Yool, E. *et al*. 1990. Proc Natl Acad Sci USA 87:1663-1667.

Revision History

Description	Section
Update information for 30-Reaction Kit	*Kit Contents and Storage* on page 7.
Change volume of Nuclease-free Water to 1.0 mL	*Kit Contents and Storage* on page 7.
Nuclease-free Water (for preparing 80% ethanol wash solution) is added	Additional Reagents and Supplies Required (Table 1.4 on page 9)
Specify dilution requirement for high concentration RNA samples.	*Determine RNA Quantity* on page 14 and *Synthesize First-Strand cDNA* on page 15.
Specify long-term storage for STOPPING POINT	Chapter 2, *Protocol*
Specify cRNA dilution requirement for concentration measurement and reaction set-up	*Assess cRNA Yield and Size Distribution* on page 19 and *Synthesize 2nd-Cycle Single-Stranded cDNA* on page 20.